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Evidence for intraspecific endocrine disruption of *Geukensia demissa* (Atlantic ribbed mussel) in an urban watershed

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ABSTRACT

Populations undergo physiological adaptations in response to environmental stressors. Our 5-year biomonitoring study of the Bronx River Estuary demonstrates comparatively low dissolved oxygen concentrations in this urbanized watershed. Additionally, our current results establish altered hormonal levels, resulting from endocrine disruption, in *Geukensia demissa* (Atlantic ribbed mussel) from the Bronx River Estuary. No studies have yet investigated a correlation between low dissolved oxygen and endocrine disruption in field-collected bivalves. Testosterone, estradiol, and progesterone levels were collected from male and female mussels in the oxygen depleted Bronx River and well-oxygenated Greenwich Cove. Bronx River mussels exhibited higher testosterone levels and lower estradiol levels than Greenwich Cove mussels. The resulting abnormal hormonal ratio seems to indicate that environmental conditions in the Bronx River facilitate an allosteric inhibition of the cytochrome P450 aromatase enzyme, which aids conversion of testosterone to estradiol. Low progesterone levels suggest that Bronx River mussels are experiencing a delay in sexual maturation, and morphometric data show a stalling of shell and tissue growth. To confirm that the mussels collected from both sites are the same species, the universal mitochondrial cytochrome c oxidase subunit I gene was analyzed, through DNA barcoding. Minimal sequential heterogeneity confirmed the mussels are the same species. Such findings suggest intraspecific divergence in various endocrine processes, resulting from environmentally induced stress.

1. Introduction

Endocrine disruption is hazardous to the vitality of any marine population. Numerous recent studies identify mechanisms and effects of endocrine disruption in invertebrates. Vertebrate steroid hormones (testosterone, estradiol, and progesterone) that are synthesized from cholesterol are found in mollusks, and evidence shows that such sex steroids can serve as ideal biomarkers of endocrine disruption (Gust et al., 2010). While the specific function of steroid hormones in mollusks' endocrine system is still speculative, reports emphasize steroids' influence on gender differentiation, gametogenesis, gonadal maturation, fertilization and embryonic development, and reproduction (Mori, 1969; Reis-Henriques and Coimbra, 1990; Matsumoto et al., 1997; Wang and Croll, 2006; Ketata et al., 2008). Specifically, testosterone and estradiol concentrations in the gonads vary during different stages in the reproduction process, largely affecting gender determination and gamete growth (Matsumoto et al., 1997; Gauthier-Clerc et al., 2006). Progesterone also influences sex specific processes, such as gametogenesis and gonadal development, and has been shown to potentially impact spawning in both sexes (Reis-Henriques and Coimbra, 1990; Wang and Croll, 2006).

Endocrine disrupting compounds are frequently found in surface water contamination, emanating from the sewage depositing of industrial facilities (Gomes and Lester, 2003; Gültekin and Ince, 2007a, 2007b). Specific analysis of wastewater content through a hepatocyte assay has shown industrially impacted water to possess estrogenic activity (Islinger et al., 1999; Gagné and Blaise, 2000). Studies have demonstrated that natural estrogens, such as 17β-estradiol, estriol and estrone, ubiquitous in certain sewage effluents can induce a feminization of fish (Björkblom et al., 2007). Certain heavy metals and chemicals stimulate endocrine disruption in mollusks. Tributyltin (TBT) acts as a neurotoxin and increases APGWamide, a neurotransmitter peptide, which releases the neurohormone Penis Morphogenic Factor (PMF) (Oberdörster and McClellan-Green, 2000; Oberdörster et al., 2005; Ketata et al., 2008). PMF generates male characteristics in both male and female mollusks. However, no study has yet explored low dissolved oxygen levels as an inducer of endocrine disruption in mollusks, or any invertebrate.

Dissolved oxygen is vital for development, reproduction, and life in nearly all aquatic organisms. The concentration of dissolved oxygen in any given body of water is dependent on a multitude of factors, including atmospheric reaeration, biochemical oxygen demand (BOD), the

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organismal rate of cellular respiration, and the rate of photosynthesis (Dobbins, 1965; Edwards and Owens, 1965; Bennett and Rathburn, 1972; Cox, 2003). Low dissolved oxygen results as a consequence of processes that consume oxygen at a greater rate than processes that produce oxygen (Rowe, 2001). In addition to natural regulators of dissolved oxygen, eutrophication can artificially reduce water's oxygen content through runoff or sewage depositing. When dissolved oxygen levels fall below 2–3 mg/L, the water is identified as hypoxic, and most organisms will struggle for survival (Diaz, 2001). Water that is borderline hypoxic remains detrimental to organisms' physiological processes (Diaz, 2001).

Urbanization and human industrial activity have a severe impact on water quality and the health of estuarine ecosystems (Limburg et al., 2005; Astaraie-Imani et al., 2012; Chin et al., 2013). Since industrialization, the Bronx River estuary, the only river to run through the city of New York, remains a site of substantial sewage contamination (Wang and Pant, 2010). The construction of the Bronx River Parkway in 1909 was the first major disruption to the surrounding natural features. This paralleling parkway reduced forest cover and contributed to poor bank stabilization as urbanization grew dense (Rachlin et al, 2007). The United States Environmental Protection Agency (EPA) labels "pathogens" as a major concern in the Bronx River, and speculates that CSOs (combined sewer overflow) are the likely source of such affliction (Crimmens, T., 2002. Bronx River Restoration: Report and Assessment). The New York City sewage system is designed to overflow into nearby rivers when waste is overly profuse. To date, the EPA has documented 31 "causes of impairment" in the Bronx River estuary, including pathogenic contamination and oxygen depletion. The EPA has yet to indicate a single "cause of impairment" for wildlife in the Greenwich Cove Estuary.

Despite various conservation efforts, our studies suggest that organisms in the Bronx River are stressed, likely from persisting adverse environmental conditions. Our research shows that the well-documented stress responding protein, heat shock protein 70 (HSP70) displays elevated baseline expression in Bronx River versus Greenwich Cove *Geukensia demissa* mussels as well as *Spartina alterniflora* marsh grass (Hans et al., 2013; Magun et al., 2013). In addition, our work indicates that Bronx River mussels serve as a valuable *in situ* model to study the potential synergistic effect of multiple active stress responders including acetylcholinesterase and heat shock proteins 70 and 90 (Magun et al., 2013). Most significantly, our laboratory continues to document lower dissolved oxygen levels in the Bronx River when compared to Greenwich Cove (Shah et al., 2012).

To establish validity for our *in situ* study of comparative hormone levels, we genetically confirmed the presence of *Geukensia demissa* species in both sites by analyzing nucleotide divergence in cytochrome c oxidase subunit I (COI) gene of the mitochondrial DNA, through DNA barcoding. Since the 1990s, several systems have emerged for the identification of species (Sugita et al., 1998; Vincent et al., 2000; Floyd et al., 2002). DNA barcoding emerged as a promising approach to make species identification more quantitative for insects, and morphologically challenging marine invertebrates, such as mollusks (Undheim et al., 2010; Keskin and Atar, 2011). Confirmation that the populations from Bronx River and Greenwich Cove are of the same species enables us to infer that divergences in endocrine ratios are responses to the environment and not due to interspecific differences.

The current study demonstrates a correlation between comparatively low dissolved oxygen levels in the Bronx River and endocrine disruption in mussels from that site. Further studies are required in order to determine the mechanism responsible for this alteration. One possibility is that low dissolved oxygen concentrations inhibit the cytochrome P450 aromatase mediated conversion of testosterone to estradiol, leading to a higher testosterone/estradiol ratio in Bronx River *Geukensia demissa* (Matthiessen and Gibbs, 1998; Alzieu, 2000; Morcillo and Porte, 2000).

Another possibility is that energy limitations mediated by low dissolved oxygen and other factors such as food limitation delay growth rate and maturation which could lead to altered levels of testosterone, estradiol, and progesterone (Franz, 1996; Petes et al., 2007).

2. Materials and methods

2.1. Water analyses

Dissolved oxygen measurements were obtained using the Winkler titration method. Oxygen was fixed at each site using manganous sulfate, alkaline potassium iodide azide, and sulfamic acid. Sodium thiosulfate was used to titrate the water sample with starch indicator to a clear endpoint. pH measurements were collected using a Flinn Scientific pH Meter (AP8673).

2.2. Sample collection and dissection

Geukensia demissa specimens were collected at low tide from Harding Lagoon in the Bronx River (40° 48′ 35.563″ N, 73° 51′ 40.893″ W), and Todd's Point in Greenwich Cove (41° 0′ 31.296″ N, 73° 34′ 18.042″ W). Our collections were performed during the months of June and July when *Geukensia demissa* ranging in size from 25 to 36 mm (width) are uniformly undergoing gametogenesis (Franz, 1996). Mussels were transported to the laboratory in buckets with water collected from the site. All samples, maintained in native water, were incubated at 4 °C and allowed 24 h to equilibrate. The length, width, and total weight of each mussel were recorded before dissection of tissues. Upon dissection, shell weight and combined gill weight were also recorded. Both gills and mantles were extracted from each organism, and stored at −20 °C.

2.3. Sex determination

Two tests were used to determine the sex of the mussels. Upon dissection, the color of the mussels' mantle was noted. In *Geukensia demissa*, a brown pigment signifies a female, and a yellowish cream color is characteristic of males (Puglisi, 2008). For confirmation, the mussel's gender was determined by the protocol of Jabbar and Davies (1987) with slight modification. One mantle from each mussel was placed in a Petri dish with 1 mL of 0.75% (w/v) thiobarbituric acid. Males developed a yellow pigment, while females developed a pink pigment. A 100% correlation was found between the results of the two gender assays.

2.4. COI gene analysis

Genetic material was isolated from a 1 by 1 mm mantle sample from 12 organisms from each site (24 total) using the QIAGEN DNeasy Blood and Tissue Kit (cat# 6904) and material was stored at −20 °C. PCR was used to amplify the COI gene using *Geukensia demissa* specific primers: 5′-CCGCGAATTAATAATTTCAGATTT-3′/5′-ACCAAAAAATCAAAATAAATGCAT-3′. Primers were designed using GenBank Sequence **FJ693154** and synthesized by Sigma Genesis Inc. in a 5 μM concentration. Twenty-five microliters of PCR reactions, composed of 2 μL template DNA and 11.5 μL of each primer in GE illustra PuReTaq Ready-To-Go PCR Beads [cat# 27-9559-01], were subsequently prepared. Reactions were run for 50 cycles with 30 s denaturation step at 94 °C, 45 s annealing step at 54 °C, and 45 s extending step at 72 °C using Techne Genius Thermo Cycler. PCR products from the 24 individuals produced amplicons of approximately 440 bp as visualized on a 2% agarose gel using pBR322/BstNI molecular weight standard from New England Biolabs [cat #N2021L]. All products, along with *Geukensia demissa* custom primers, were sent to Genewiz Inc. for sequencing. Bronx River and Greenwich Cove sequences were aligned by Nucleotide BLAST, and percent similarity was ascertained using the CLUSTAL W alignment tool (Thompson et al., 1994; Ni et al., 2012).

2.5. Testosterone, estradiol, and progesterone enzyme immunoassay (EIA)

Per each assay, one gill from each mussel was homogenized in 1.2 mL ice-cold 50:50 water:methanol. Homogenates were extracted three times with 5 mL of high purity diethyl ether for the testosterone assay, or 5 mL of dichloromethane for the estradiol and progesterone assay. At room temperature, organic extracts were evaporated to dryness. EIA buffer (500 μL; Cayman Chemical, Ann Arbor, MI, USA) was used to reconstitute each sample.

Steroid hormones were quantified using commercial testosterone, estradiol, and progesterone enzyme immunoassay kits (Cayman Chemical, cat#582701, 582251, 582601). The assay is based on the competitive immunoreactions between the free hormone and steroid linked acetylcholinesterase (AChE) conjugate for a finite amount of antiserum. EIAs were performed in 96-well plate format, with each plate being read by a Fisher Scientific* Multiskan* FC Microplate Reader (cat# 14-387-360). Standards, provided in each kit, were diluted to form standard curves and assayed in duplicate on each plate. Testosterone standard concentrations range from 500 to 3.9 pg/mL, estradiol standard concentrations range from 6.6 to 4000 pg/mL, and progesterone standard concentrations range from 7.8 to 1000 pg/mL. Two blank, two non-specific binding, three maximum binding, and one total activity well(s) were also run on each plate. Samples were run in triplicate at three dilutions: 1:1, 1:2, and 1:10. The intra- and inter-assay coefficients of variation (CVs) were less than 10%. Cross reactivity of the testosterone antiserum with various steroids is as follows: testosterone 100%, 5α-dihydrotestosterone 27.4%, 5β-dihydrotestosterone 18.9%, methyltestosterone 4.7%, androstenedione 3.7%, 11-keto testosterone 2.2%, and less than 1% for all other steroids tested (Cayman Chemicals). Cross reactivity of the estradiol antiserum with various steroids is as follows: estradiol 100%, estradiol-3-sulfate 14.5%, estradiol-3-glucuronide 14%, estrone 12%, estradiol-17 glucuronide 10%, and less than 0.5% for all other steroids tested (Cayman Chemicals). Cross reactivity of the progesterone antiserum with various steroids is as follows: progesterone 100%, 17β-estradiol 7.2%, 5β-Pregnan-3α-ol-20-one 6.7%, pregnenolone 2.5%, and less than 1% for all other steroids tested (Cayman Chemicals).

2.6. Statistical analyses

P-values for water data, morphometric data, and steroid hormone concentrations were determined through a *t*-test. Any *p*-value less than or equal to 0.05 is considered statistically significant.

3. Results

3.1. Water analyses

Bronx River water has consistently possessed lower dissolved oxygen concentrations than Greenwich Cove over our 5-year study. In the past 2 years, our show data has shown decreasing dissolved oxygen concentrations in the Bronx River as a trend. Difference between the temperature and pH of the water at the two sites was minimal (Table 1).

Table 1
Average levels of dissolved oxygen levels (mg/L), water temperature (°C), and pH, over a 5-year survey of the Bronx River and Greenwich Cove.

Site	Dissolved oxygen (mg/L)	Temperature (°C)	pH
Bronx River	3.9 ± 0.2	22.35 ± 0.24	7.29 ± 0.02
Greenwich Cove	10.0 ± 0.7	25.32 ± 0.94	7.97 ± 0.07

All water collections (3 each year) occurred in June and July, around low tide. Data are presented as mean ± SEM ($n = 15$). $p < 0.0001$ for dissolved oxygen concentrations.

3.2. Morphometric data

Bronx River mussels were consistently of smaller size (length/width) and lower weight than Greenwich Cove mussels over our 5-year study. Average combined gill weight was also significantly lower in Bronx River mussels.

3.3. COI gene analysis

Among Bronx River mussels, CLUSTAL W alignment revealed negligible heterogeneity (0–1%) in COI sequence. CLUSTAL W alignment also showed insignificant (0–1%) divergence in COI sequence within the Greenwich Cove population. When compared across sites, however, alignment disclosed a consistent 2% sequential heterogeneity. Organism gender appeared to have no impact on COI gene sequence.

3.4. Tissue steroid levels

Steroid levels were determined in gill tissue. The same mussel test cohort was used for the testosterone and estradiol assays, but different individuals were used for progesterone assay. Testosterone concentrations were significantly higher ($p < 0.05$) in Bronx River mussels relative to Greenwich Cove mussels (2.1 fold in males, 3.6 fold in females) (Fig. 1A). In both sites, male mussels contained higher levels of testosterone than female mussels. The Bronx River mussels also possessed

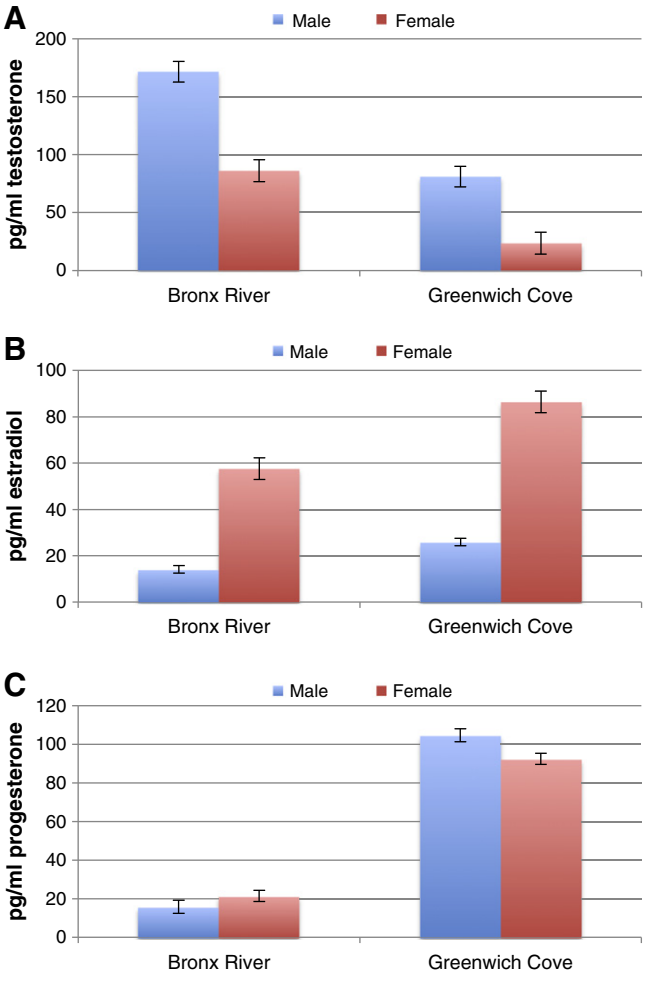

Fig. 1. Gill steroid hormone concentrations (pg/mL) for (A) testosterone, (B) estradiol, and (C) progesterone. Male and female distinctions are presented. Data are shown as mean ± SEM ($n = 16$, 8 males, 8 females). $p = 0.00885$ for testosterone, $p = 0.04175$ for estradiol, and $p = 0.0001$ for progesterone.

lower estradiol concentrations than Greenwich Cove mussels (Fig. 1B). Female mussels had higher tissue concentrations of estradiol than male mussels at the Bronx River (57.6 ± 1.6 pg/mL versus 14.1 ± 0.8 pg/mL) and Greenwich Cove (86.4 ± 3.1 pg/mL versus 25.9 ± 2.5 pg/mL). Thus, the testosterone/estradiol ratio (combining concentrations in male and female mussels) is significantly higher in Bronx River mussels (3.60) than Greenwich Cove mussels (0.93). Progesterone levels were not influenced by gender, but varied between the two sites. The concentration was higher in gill tissue of Greenwich Cove mussels than Bronx River mussels (6.6- and 4.3-fold for male and female, respectively) (Fig. 1C).

4. Discussion

Results of our comparative steroid analysis suggest that mussels living in an urbanized and environmentally impacted watershed are experiencing endocrine disruption, indicated by the relative concentrations of all three endogenous steroids (testosterone, estradiol, and progesterone). The Bronx River mussels have significantly higher testosterone levels (2.1 fold in males, 3.6 fold in females) and significantly lower estradiol concentrations (1.8 fold in males, 1.5 fold in females) (Fig. 1A). The average ratio of testosterone/estradiol (pg/mL) is 3.60 in Bronx River mussels, juxtaposed to the normal 0.93 in Greenwich Cove mussels.

Our findings concur with Friesen et al. (2012) and other recent studies that correlate low dissolved oxygen with endocrine disruption in fish species (Wu et al., 2003; Landry et al., 2007). The pathway for such an alteration was identified as a hypoxia mediated inhibition of cytochrome P450 aromatase activity (Alzieu, 2000; Matthiessen and Gibbs, 1998; Morcillo and Porte, 2000; Shang et al., 2006). This mechanism may explain our observed alterations of the testosterone/estradiol ratio in mussels from the oxygen depleted Bronx River. Such an inhibition could lead to a general masculinization of both male and female mussels, though more research is required.

Evidence also shows that low dissolved oxygen can impact growth and development, RNA/DNA ratio, gonad and embryo formation, fitness, and survival rates in fish species (Zhou et al., 2001; Gercken et al., 2006; Hassell et al., 2008; Kolding et al., 2008; Wang et al., 2008). These findings correlate well with our 5-year morphometric studies demonstrating that *Geukensia demissa* from the hypoxic Bronx River site have a consistently lower body mass index than ribbed mussels from Greenwich Cove, CT.

In addition, studies show that heavy metals, specifically tributyltin (TBT) inhibit cytochrome P450 aromatase, thus blocking the conversion of testosterone to estradiol, and affecting the testosterone/estradiol ratio (Matthiessen and Gibbs, 1998; Alzieu, 2000; Morcillo and Porte, 2000). Other substances known to induce endocrine disruption and the conversion of testosterone to estradiol in marine invertebrates include: herbicides (diquat dibromide, atrazine, simazine, diuron), metals (cadmium, selenium, zinc, mercury, lead), PCBs, alkylphenols (nonylphenol, pentylphenol), and insecticides (DDT, ednrin, toxaphene, piperonyl butoxide, methoprene) (Depledge and Billinghurst, 1999).

Endocrine disruption is also indicated by the significantly low level of progesterone in the gill tissue of Bronx River mussels averaging 18.6 ± 1.3 pg/mL, as compared to an average of 98.6 ± 1.9 pg/mL in Greenwich Cove mussels. Conditions in the Bronx River could potentially alter energy demands of the mussels, forcing them to distribute the limited available oxygen towards aerobic metabolic processes, rather than the synthesis of progesterone. Our conclusion that progesterone levels are not gender mediated supports the hypothesis of Reis-Henriques and Coimbra (1990), which indicates that male and female mussels require a similar pattern and amount of progesterone throughout their reproductive cycle. We expect the progesterone deficiency to have other physiological effects. During the maturation of oocytes in *M. edulis*, an increase in lysosomal enzyme activity is usually noted (Peek and Gabott, 1990). In correlation, rising progesterone levels in *M. edulis* have been shown to result in the destabilization of lysosomes (Moore et al., 1978). Thus, studies suggest progesterone as a biomarker of sexual maturation, in regard to oocyte progression (Siah et al., 2003). Moreover, studies show that progesterone levels peak during active gametogenesis, when the male's gonads are maturing and females are in the spawning stage (Reis-Henriques and Coimbra, 1990; Siah et al., 2002). Due to a lack of progesterone, the Bronx River mussels may be undergoing a deceleration of the rate of sexual maturation and oocyte development. In addition, progesterone has shown to act as a gonad messenger for neurohormones (Siah et al., 2002). The Bronx River mussels may be experiencing a delay in germ cell proliferation, and our results lead us to hypothesize that the lack of progesterone has affected the production of neurohormones. To confirm this hypothesis, further studies will be required to examine neurohormone-receptor responses to oxygen depletion.

COI gene analysis revealed only slight heterogeneity for organisms from the same site (0–1% Bronx, 0–1% Greenwich), thus indicating that mussel populations at both sites contain only one species. The 2% COI sequence divergence when comparing mussels across sites indicates that mussels at both sites are of the same species (*Geukensia demissa*). This supports the claims of Hebert et al. (2003) that for two populations, intraspecific COI sequence divergence is approximately 2% (Avise, 2000; Cognato, 2006). The consistent 2% heterogeneity in COI sequence across sites indicates adaptive pressure, indicating the possibility of allopatric speciation in the future.

As mentioned above, morphometric analysis demonstrates a delayed shell growth and tissue growth in Bronx River mussels (Table 2). The growth rate of mussels tends to vary by season, higher in spring and summer, and lower in winter (Page and Hubbard, 1987; Garen et al., 2004). In addition, shell and tissue growth are generally affiliated with the reproductive cycle of the given population (Bayne and Worrall, 1980; Handå et al., 2011). However, in mussels, shell growth is not directly correlated to tissue growth (Hilbish, 1986; Kautsky, 1982; Rodhouse et al., 1984). Environmental stress and food limitations are known to impede growth processes. The 31 causes of impairment (as documented by the EPA) or the limitation of dissolved oxygen in the Bronx River may require that mussels conserve energy for vital survival processes, which would delay shell and tissue growth. A recent study transplanted *Mytilus galloprovincialis* and *Perna canaliculus* to high-

Table 2
Average body mass, condition index (CI) total ((wet weight / total weight) × 100), CI shell ((wet weight / shell weight) × 100), and combined gill weight in *Geukensia demissa* mussels taken over a 5-year span from the Bronx River and Greenwich Cove (Shah et al., 2012).

	Bronx River					Greenwich Cove				
	2009	2010	2011	2012	2013	2009	2010	2011	2012	2013
Length/Width (mm)	66.6/28.4	60.0/24.0	62.0/27.3	ND	72.8/30.9	77.0/31.1	85.0/31.0	81.1/32.6	ND	86.2/40.2
Average body mass (g)	17.5	18.16	20.39	30.1	37.80	21.8	35.88	36.03	39.7	46.76
Condition index total	44	41	48	38	42	41	44	51	43	58
Condition index shell	84	72	89	63	79	74	80	106	76	89
Average combined gill mass (g)	0.87	1.08	0.80	ND	0.97	1.33	1.30	1.13	ND	1.21

ND = no data available.
$p = 0.0099$ for length, and $p = 0.0150$ for average combined gill mass.

stress and low-stress elevation edges of an intertidal mussel bed to compare growth and reproduction (Petes et al., 2007). Mussels living in the high-stress edge exhibited reduced growth rate and a smaller tissue mass (Petes et al., 2007). Additional studies demonstrate endogenous steroid levels as regulators of growth. In the fresh water mussel, *Elliptio complanata* (Gagné et al., 2001) demonstrated that high estrogen levels promote total and soft tissue weights. The lack of estradiol in Bronx River mussels may have stunted tissue growth, accounting for the reduced average total body weight and average combined gill weight (Table 2).

5. Conclusion

Overall, this study is the first to demonstrate a correlation between low dissolved oxygen emanating from an urbanized site and disruption in endogenous steroid levels, physiological development, and sexual maturation in *Geukensia demissa.*

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